

## FINAL REPORT

Study Title

**EVALUATION OF THE MUTAGENIC ACTIVITY OF MLA-3202 IN  
THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY  
AND THE *ESCHERICHIA COLI* REVERSE MUTATION ASSAY**

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Laboratory Project Identification

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## 2. STATEMENT OF GLP COMPLIANCE

WIL Research Europe B.V., 's-Hertogenbosch, The Netherlands

All phases of this study performed by the test facility were conducted in compliance with the following GLP regulations:

- OECD Principles of Good Laboratory Practice concerning Mutual Acceptance of Data in the Assessment of Chemicals, 26 November 1997 (C(97) 186 Final);
- EC Council Directive 2004 (2004/10/EC, February 11, 2004, Official Journal of February 20, 2004).

Except for the following:

- The quality environment in which the characterisation of the test item was performed was not known.

Analysis of test item in vehicle for concentration, stability, homogeneity was not performed, however, to limit the impact, the test item preparation was performed with approved procedures and documented in detail. Preparations were visually inspected for homogeneity prior to use and all preparations were used within 2.5 hours after adding vehicle to the test item.

The data generated and reported are considered to be valid.

WIL Research Europe B.V.

Signature: \_\_\_\_\_



Name: \_\_\_\_\_

C.M. Verspeek-Rip

Title: \_\_\_\_\_

Study Director

Date: \_\_\_\_\_

26 May 2016

**3. TEST FACILITY QUALITY ASSURANCE STATEMENT**

WIL Research Europe B.V., 's-Hertogenbosch, The Netherlands.

Study title: Evaluation of the mutagenic activity of MLA-3202 in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay.

This report was inspected by the WIL Research Europe Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s). The reported method and procedures were found to describe those used and the report reflects the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

**Project** 511879

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
<b>Study</b>	Study Plan	24-Mar-2016	24-Mar-2016	24-Mar-2016
	Report	19-May-2016	19-May-2016	19-May-2016
<b>Process</b>	<b>Test Substance Receipt</b> Test Substance Handling	08-Feb-2016	29-Feb-2016	01-Mar-2016
	<b>Genetic and In Vitro Toxicology</b> Test Substance Handling Exposure Observations/Measurements Specimen Handling	22-Mar-2016	31-Mar-2016	04-Apr-2016

The facility inspection program is conducted in accordance with Standard Operating Procedure.

The review of the final report was completed on the date of signing this QA statement.

WIL Research Europe B.V.

Signature: 

Name: C. Mitchell B.Sc., FRQA  
Head of Quality Assurance

Date: 

#### 4. SUMMARY

Evaluation of the mutagenic activity of MLA-3202 in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay.

MLA-3202 was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* (WP<sub>2</sub>uvrA). The test was performed in two independent experiments in the presence and absence of S9-mix (rat liver S9-mix induced by Aroclor 1254). An additional experiment was performed with tester strain TA98 in the absence of S9-mix, and TA100 in the absence and presence of S9-mix.

The study procedures described in this report were based on the most recent OECD and EC guidelines.

Batch RC-1045 of MLA-3202 was a clear amber-red liquid. The test item was dissolved in dimethyl sulfoxide.

In the dose range finding test, the test item was tested up to concentrations of 5000 µg/plate in the absence and presence of S9-mix in the strains TA100 and WP<sub>2</sub>uvrA. MLA-3202 precipitated on the plates at the dose level of 5000 µg/plate. In tester strain TA100, toxicity was observed in the absence and presence of S9-mix. In tester strain WP<sub>2</sub>uvrA, no toxicity was observed at any of the dose levels tested. Results of this dose range finding test were reported as part of the first mutation assay.

Based on the results of the dose range finding test, the test item was tested in the first mutation assay at a concentration range of 52 to 5000 µg/plate in the absence and presence of 5% (v/v) S9-mix in the tester strains TA1535, TA1537 and TA98. The test item precipitated on the plates at the top dose of 5000 µg/plate. The bacterial background lawn was not reduced at any of the concentrations tested and no biologically relevant decrease in the number of revertants was observed.

In a follow-up experiment of the assay with additional parameters, the test item was tested at a concentration range of 492 to 5000 µg/plate in the absence and presence of 10% (v/v) S9-mix in the tester strains TA1535, TA1537, TA98, TA100 and WP<sub>2</sub>uvrA. The test item precipitated on the plates at the top dose of 5000 µg/plate. Cytotoxicity, as evidenced by a decrease in the number of revertants, was observed in several tester strains, TA1535 (presence of S9-mix), TA1537 (absence of S9-mix), TA98 (absence of S9-mix) and TA100 (absence and presence of S9-mix).

In the second mutation test, not enough non-toxic dose levels were present in the tester strains TA98 (absence of S9-mix) and TA100 (absence and presence of S9-mix), therefore an additional experiment was performed. In this mutation experiment, the test item was tested at a concentration range of 17 to 5000 µg/plate in the absence of S9-mix in both tester strains, and in the presence of 10% (v/v) S9-mix in tester strain TA100. The test item precipitated on the plates at dose level of 5000 µg/plate. In tester strain TA100, toxicity was observed in the absence and presence of S9-mix. In tester strain TA98, no toxicity was observed at any of the dose levels tested.

MLA-3202 did not induce a significant dose-related increase in the number of revertant (His<sup>+</sup>) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp<sup>+</sup>) colonies in the tester strain WP<sub>2</sub>uvrA both in the absence and presence of S9-metabolic activation. These results were confirmed in a follow-up experiment.

In this study, acceptable responses were obtained for the negative and strain-specific positive control items indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that MLA-3202 is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

## 5. INTRODUCTION

### 5.1. Study schedule

Experimental starting date : 04 April 2016  
Experimental completion date : 28 April 2016

### 5.2. Purpose

The objective of this study was to evaluate MLA-3202 for its ability to induce reverse mutations in a gene of histidine-requiring *Salmonella typhimurium* bacterial strains resulting in histidine-independent strains, and in a gene of tryptophan-requiring *Escherichia coli* bacterial strain resulting in a tryptophan-independent strain.

#### Background of the test system

The *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay have been shown to be rapid and adequate indicators for the mutagenic activity of a wide range of chemical compounds.

The assay was conducted in the absence and presence of a metabolizing system (S9-mix).

The *Salmonella typhimurium* strains used in this study were TA1535, TA1537, TA98 and TA100. The *Escherichia coli* strain used was WP<sub>2uvrA</sub>. The strains TA1537 and TA98 are capable of detecting frameshift mutagens, strains TA1535, TA100 and WP<sub>2uvrA</sub> are capable of detecting base-pair substitution mutagens (1-5).

### 5.3. Guidelines

The study procedures described in this report are in compliance with the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals; Guideline no. 471: "Genetic Toxicology: Bacterial Reverse Mutation Test" (Adopted July 21, 1997).
- European Community (EC). Commission regulation (EC) No. 440/2008, Part B: Methods for the Determination of Toxicity and other health effects, Guideline B.13/14: "Mutagenicity: Reverse Mutation Test using Bacteria". Official Journal of the European Union No. L142, 31 May 2008.

### 5.4. Retention of records and materials

Records and material pertaining to the study, which include study plan and amendments, raw data and the final report will be retained in the archives of the test facility for a minimum of 5 years after the finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. The test facility will retain information concerning decisions made.

A sample of the test item will be retained until expiry date or applicable retest date. After this period the sample(s) will be destroyed.

### 5.5. Responsible personnel

#### 5.5.1. Test facility

Study Director : C.M. Verspeek-Rip

#### 5.5.2. Sponsor Representative

Study Monitor : Audrey Batoon, Ph.D.

## 6. MATERIALS AND METHODS

### 6.1. Test item

#### 6.1.1. Test item information

Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

For Certificate of Analysis, see [APPENDIX 6](#).

#### 6.1.2. Study specific test item information

Purity/composition correction factor	No correction factor required
Test item handling	No specific handling conditions required
Stability at higher temperatures	Stable
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3

### 6.2. Vehicle information

Stability in vehicle	Dimethyl sulfoxide: Not indicated
Solubility in vehicle	Dimethyl sulfoxide: Not indicated

### 6.3. Reference item

#### 6.3.1. Vehicle control

The vehicle of the test item, which was dimethyl sulfoxide (SeccoSolv, Merck, Darmstadt, Germany).

#### 6.3.2. Positive controls

##### Without metabolic activation (-S9-mix):

<u>Strain</u>	<u>Chemical</u>	<u>Concentration/plate</u>	<u>Solvent</u>
TA1535	sodium azide (SA) (Sigma Aldrich Chemie, Steinheim, Germany)	5 µg	Saline
TA1537	ICR-191 (Sigma)	2.5 µg	DMSO
TA98	2-nitrofluorene (NF) (Sigma)	10 µg	DMSO
TA100	methylmethanesulfonate (MMS) (Sigma)	650 µg	DMSO
WP <sub>2uvrA</sub>	4-nitroquinoline N-oxide (4-NQO) (Sigma)	10 µg	DMSO

##### With metabolic activation (+S9-mix):

The positive control item used for all tester strains was 2-aminoanthracene (2AA) (Sigma). The following concentrations were used:

<u>Strain</u>	<u>Concentration/plate</u>	<u>Amount of S9-mix</u>	<u>Solvent</u>
TA1535	2.5 µg	5 and 10%	DMSO
TA1537	2.5 µg	5%	DMSO
TA1537	5 µg	10%	DMSO
TA98	1 µg	5 and 10%	DMSO
TA100	1 µg	5%	DMSO
TA100	2 µg	10%	DMSO
WP <sub>2uvrA</sub>	15 µg	5 and 10%	DMSO

Solvents for reference items

Saline = physiological saline (Eurovet Animal Health, Bladel, The Netherlands)

DMSO = dimethyl sulfoxide (Merck)

**6.4. Test item preparation**

No correction was made for the purity/composition of the test item. A solubility test was performed. MLA-3202 was dissolved in dimethyl sulfoxide.

**6.5. Test system**

Test system	<i>Salmonella typhimurium</i> bacteria and <i>Escherichia coli</i> bacteria
Rationale	Recommended test system in international guidelines (e.g. OECD, EC).
Source	Trinova Biochem GmbH, Germany [Master culture from Dr. Bruce N. Ames (TA1535: 2006, TA1537: 2009, TA98: 2015, TA100: 2015; and Master culture from The National Collections of Industrial and Marine Bacteria, Aberdeen, UK (WP <sub>2</sub> uvrA: 2008)]

The characteristics of the different *Salmonella typhimurium* strains were as follows:

<u>Strain</u>	<u>Histidine mutation</u>	<u>Mutation type</u>
TA1537	<i>hisC3076</i>	Frameshift
TA98	<i>hisD3052/R-factor*</i>	Frameshift
TA1535	<i>hisG46</i>	Base-pair substitutions
TA100	<i>hisG46/R-factor*</i>	Base-pair substitutions

\*: R-factor = plasmid pKM101 (increases error-prone DNA repair)

Each tester strain contained the following additional mutations:

<u>rfa</u>	: deep rough (defective lipopolysaccharide cellcoat)
<u>gal</u>	: mutation in the galactose metabolism
<u>chl</u>	: mutation in nitrate reductase
<u>bio</u>	: defective biotin synthesis
<u>uvrB</u>	: loss of the excision repair system (deletion of the ultraviolet-repair B gene)

The *Salmonella typhimurium* strains are regularly checked to confirm their histidine-requirement, crystal violet sensitivity, ampicillin resistance (TA98 and TA100), UV-sensitivity and the number of spontaneous revertants.

The *Escherichia coli* WP<sub>2</sub>uvrA strain detects base-pair substitutions. The strain lacks an excision repair system and is sensitive to agents such as UV. The sensitivity of the strain to a wide variety of mutagens has been enhanced by permeabilization of the strain using Tris-EDTA treatment (Ref.1). The strain is regularly checked to confirm the tryptophan-requirement, UV-sensitivity and the number of spontaneous revertants.

Stock cultures of the five strains were stored in liquid nitrogen (-196°C).

**6.6. Cell culture**Preparation of bacterial cultures

Samples of frozen stock cultures of bacteria were transferred into enriched nutrient broth (Oxoid LTD, Hampshire, England) and incubated in a shaking incubator (37 ± 1°C, 150 rpm), until the cultures reached an optical density of 1.0 ± 0.1 at 700 nm (10<sup>9</sup> cells/ml). Freshly grown cultures of each strain were used for testing.

### Agar plates

Agar plates (ø 9 cm) containing 25 ml glucose agar medium. Glucose agar medium contained per liter: 18 g purified agar (Oxoid LTD) in Vogel-Bonner Medium E, 20 g glucose (Fresenius Kabi, Bad Homburg, Germany). The agar plates for the test with the *Salmonella typhimurium* strains also contained 12.5 µg/plate biotin (Merck) and 15 µg/plate histidine (Sigma) and the agar plates for the test with the *Escherichia coli* strain contained 15 µg/plate tryptophan (Sigma).

### Top agar

Milli-Q water containing 0.6% (w/v) bacteriological agar (Oxoid LTD) and 0.5% (w/v) sodium chloride (Merck) was heated to dissolve the agar. Samples of 3 ml top agar were transferred into 10 ml glass tubes with metal caps. Top agar tubes were autoclaved for 20 min at  $121 \pm 3^\circ\text{C}$ .

### Environmental conditions

All incubations were carried out in a controlled environment at a temperature of  $37.0 \pm 1.0^\circ\text{C}$  (actual range  $34.9 - 39.8^\circ\text{C}$ ). The temperature was continuously monitored throughout the experiment. Due to addition of plates (which were at room temperature) to the incubator or due to opening and closing the incubator door, temporary deviations from the temperature may occur. Based on laboratory historical data these deviations are considered not to affect the study integrity.

## **6.7. Metabolic activation system**

Rat liver microsomal enzymes (S9 homogenate) were obtained from Trinova Biochem GmbH, Giessen, Germany and were prepared from male Sprague Dawley rats that had been injected intraperitoneally with Aroclor 1254 (500 mg/kg body weight).

Each S9 batch is characterised with the mutagens benzo-(a)-pyrene and 2-aminoanthracene, which require metabolic activation, in tester strain TA100 at concentrations of 5 µg/plate and 2.5 µg/plate, respectively.

### **6.7.1. Preparation of S9-mix**

S9-mix was prepared immediately before use and kept on ice. S9-mix contained per 10 ml: 30 mg NADP (Randox Laboratories Ltd., Crumlin, United Kingdom) and 15.2 mg glucose-6-phosphate (Roche Diagnostics, Mannheim, Germany) in 5.5 ml Milli-Q water (first experiment) and 5.0 ml Milli-Q water (second and third experiment) (Millipore Corp., Bedford, MA., USA); 2 ml 0.5 M sodium phosphate buffer pH 7.4; 1 ml 0.08 M  $\text{MgCl}_2$  solution (Merck); 1 ml 0.33 M KCl solution (Merck). The above solution was filter (0.22 µm)-sterilized. To 9.5 ml of S9-mix components 0.5 ml S9-fraction was added (5% (v/v) S9-fraction) to complete the S9-mix in the first experiment and to 9.0 ml of S9-mix components 1.0 ml S9-fraction was added (10% (v/v) S9-fraction) to complete the S9-mix in the second experiment.

## **6.8. Study design**

### **6.8.1. Dose range finding test**

Selection of an adequate range of doses was based on a dose range finding test with the strains TA100 and WP<sub>2</sub>uvrA, both with and without 5% (v/v) S9-mix. Eight concentrations, 1.7, 5.4, 17, 52, 164, 512, 1600 and 5000 µg/plate were tested in triplicate. The highest concentration of MLA-3202 used in the subsequent mutation assay was 5000 µg/plate.

### **6.8.2. Mutation assay**

At least five different doses (increasing with approximately half-log steps) of the test item were tested in triplicate in each strain. The above mentioned dose range finding study with the two tester strains TA100 and WP<sub>2</sub>uvrA, is reported as a part of the first mutation experiment. In the second part of this experiment, the test item was tested both in the absence and presence of 5% (v/v) S9-mix in the tester strains TA1535, TA1537 and TA98. In a follow-up experiment with additional parameters, the test item

was tested both in the absence and presence of 10% (v/v) S9-mix in all tester strains. An additional experiment was performed with tester strain TA98 in the absence of S9-mix, and TA100 in the absence and presence of 10% (v/v) S9-mix.

The negative control (vehicle) and relevant positive controls were concurrently tested in each strain in the presence and absence of S9-mix.

Top agar in top agar tubes was melted by heating to  $45 \pm 2^\circ\text{C}$ . The following solutions were successively added to 3 ml molten top agar: 0.1 ml of a fresh bacterial culture ( $10^9$  cells/ml) of one of the tester strains, 0.1 ml of a dilution of the test item in DMSO and either 0.5 ml S9-mix (in case of activation assays) or 0.5 ml 0.1 M phosphate buffer (in case of non-activation assays). The ingredients were mixed on a Vortex and the content of the top agar tube was poured onto a selective agar plate. After solidification of the top agar, the plates were inverted and incubated in the dark at  $37.0 \pm 1.0^\circ\text{C}$  for  $48 \pm 4$  h. After this period revertant colonies (histidine independent (His<sup>+</sup>) for *Salmonella typhimurium* bacteria and tryptophan independent (Trp<sup>+</sup>) for *Escherichia coli*) were counted.

### 6.8.3. Colony counting

The revertant colonies were counted automatically with the Sorcerer Colony Counter. Plates with sufficient test item precipitate to interfere with automated colony counting were counted manually. Evidence of test item precipitate on the plates and the condition of the bacterial background lawn were evaluated when considered necessary, macroscopically and/or microscopically by using a dissecting microscope.

## 6.9. Interpretation

### 6.9.1. Acceptability of the assay

A *Salmonella typhimurium* reverse mutation assay and/or *Escherichia coli* reverse mutation assay is considered acceptable if it meets the following criteria:

- The vehicle control and positive control plates from each tester strain (with or without S9-mix) must exhibit a characteristic number of revertant colonies when compared against relevant historical control data generated at WIL Research Europe.
- The selected dose range should include a clearly toxic concentration or should exhibit limited solubility as demonstrated by the preliminary toxicity range-finding test or should extend to 5 mg/plate.
- No more than 5% of the plates are lost through contamination or some other unforeseen event. If the results are considered invalid due to contamination, the experiment will be repeated.

### 6.9.2. Data evaluation and statistical procedures

No formal hypothesis testing was done.

In addition to the criteria stated below, any increase in the total number of revertants should be evaluated for its biological relevance including a comparison of the results with the historical control data range.

A test item is considered negative (not mutagenic) in the test if:

- The total number of revertants in the tester strain TA100 or WP<sub>2</sub>uvrA is not greater than two (2) times the concurrent control, and the total number of revertants in tester strains TA1535, TA1537 or TA98 is not greater than three (3) times the concurrent vehicle control.
- The negative response should be reproducible in at least one follow-up experiment.

A test item is considered positive (mutagenic) in the test if:

- The total number of revertants in the tester strain TA100 or WP<sub>2</sub>uvrA is greater than two (2) times the concurrent control, or the total number of revertants in tester strains TA1535, TA1537, TA98 is greater than three (3) times the concurrent vehicle control.

- b) In case a follow up experiment is performed when a positive response is observed in one of the tester strains, the positive response should be reproducible in at least one follow up experiment.

## 6.10. List of deviations

### 6.10.1. List of study plan deviations

There were no deviations from the study plan.

### 6.10.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

## 7. ELECTRONIC SYSTEMS FOR DATA ACQUISITION

The following electronic systems were used for data acquisition:

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA).
- Ames study Manager version 1.23 (Perceptive Instruments Ltd., St Edmunds, Suffolk, United Kingdom).

## 8. RESULTS

### 8.1. Dose range finding test/first mutation experiment

MLA-3202 was tested in the tester strains TA100 and WP<sub>2uvrA</sub> at concentrations of 1.7, 5.4, 17, 52, 164, 512, 1600 and 5000 µg/plate in the absence and presence of S9-mix. Based on the results of the dose range finding test, the following dose range was selected for the first mutation experiment with the tester strains, TA1535, TA1537 and TA98 in the absence and presence of S9-mix: 5.4, 17, 52, 164, 512, 1600 and 5000 µg/plate.

The results are shown in [Table 1](#) and [Table 2](#). The individual data are presented in [APPENDIX 3](#).

#### Precipitate

Precipitation of MLA-3202 on the plates was observed at the start of the incubation period at concentrations of 1600 µg/plate and 5000 µg/plate and at 5000 µg/plate at the end of the incubation period.

#### Toxicity

To determine the toxicity of MLA-3202, the reduction of the bacterial background lawn, the increase in the size of the microcolonies and the reduction of the revertant colonies were examined. The definitions are stated in [APPENDIX 2](#).

Cytotoxicity, as evidenced by a decrease in the number of revertants, was observed in tester strain TA100 in the absence and presence of S9-mix.

In the tester strains TA1535 (absence of S9-mix), TA1537 (absence and presence of S9-mix) and TA98 (absence of S9-mix), fluctuations in the number of revertant colonies below the laboratory historical control data range were observed. However, since no dose-relationship was observed, these reductions are not considered to be caused by toxicity of the test item. It is more likely these reductions are caused by an incidental fluctuation in the number of revertant colonies.

#### Mutagenicity

No increase in the number of revertants was observed upon treatment with MLA-3202 under all conditions tested.

## 8.2. Experiment 2

To obtain more information about the possible mutagenicity of MLA-3202, a second mutation experiment was performed in the absence of S9-mix and in the presence of 10% (v/v) S9-mix. Based on the results of the first mutation assay, the test item was tested up to the dose level of 5000 µg/plate in strains TA1535, TA1537, TA98, TA100 and WP<sub>2</sub>uvrA. The results are shown in [Table 3](#), the individual data are presented in [APPENDIX 3](#).

### Precipitate

Precipitation of MLA-3202 on the plates was observed at the start of the incubation period at concentrations of 1568 µg/plate and upwards and at 5000 µg/plate at the end of the incubation period.

### Toxicity

In tester strain WP<sub>2</sub>uvrA, no reduction of the bacterial background lawn and no biologically relevant decrease in the number of revertants was observed at any of the concentrations was observed in the absence and presence of S9-mix.

Cytotoxicity, as evidenced by a decrease in the number of revertants, was observed in the tester strains TA1537 and TA98 in the absence of S9-mix, in tester strain TA1535 in the presence of S9-mix and in tester strain TA100 in the absence and presence of S9-mix.

In strain WP<sub>2</sub>uvrA and TA1535, both in the absence of S9-mix, fluctuations in the number of revertant colonies below the laboratory historical control data range were observed. However, since no dose-relationship was observed, these reductions are not considered to be caused by toxicity of the test item. It is more likely these reductions are caused by incidental fluctuations in the number of revertant colonies.

### Mutagenicity

In the second mutation assay, no increase in the number of revertants was observed upon treatment with MLA-3202 under all conditions tested.

## 8.3. Experiment 3

Since in the second mutation test, not enough non-toxic dose levels were present in the tester strains TA98 (absence of S9-mix) and TA100 (absence and presence of S9-mix), an additional experiment was performed. In this third mutation experiment, the following lower dose range was tested: 17, 52, 164, 512, 1600 and 5000 µg/plate. The results are shown in [Table 4](#), the individual data are presented in [APPENDIX 3](#).

### Precipitate

Precipitation of MLA-3202 on the plates was observed at the start of the incubation period at concentrations of 1600 and 5000 µg/plate and at 5000 µg/plate at the end of the incubation period.

### Toxicity

Cytotoxicity, as evidenced by a decrease in the number of revertants, was observed in tester strain TA100 in the absence and presence of S9-mix.

In tester strain TA98, no reduction of the bacterial background lawn and no biologically relevant decrease in the number of revertants was observed at any of the concentrations tested.

### Mutagenicity

In the third mutation assay, no increase in the number of revertants was observed upon treatment with MLA-3202 under all conditions tested.

## 9. DISCUSSION AND CONCLUSION

All bacterial strains showed negative responses over the entire dose range, i.e. no significant dose-related increase in the number of revertants in two experiments.

The negative control values were within the laboratory historical control data ranges.

The strain-specific positive control values were within the laboratory historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly, except the response for TA98 in the absence of S9-mix, third experiment. The purpose of the positive control is as a reference for the test system, where a positive response is required to check if the test system functions correctly. Since the value was more than 3 times greater than the concurrent solvent control values, this deviation in the mean plate count of the positive control had no effect on the validity of the study.

Based on the results of this study it is concluded that MLA-3202 is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

## 10. REFERENCES

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- 4 Green, M.H.L. and Muriel, W.J., 1976, Mutagen testing using Trp<sup>+</sup> reversion in *Escherichia coli*, Mutation Res., 38, 3-32.
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## APPENDIX 1 SUMMARY TABLES

**Table 1** Dose range finding test: Mutagenic response of MLA-3202 in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay

Dose ( $\mu\text{g}/\text{plate}$ )	Mean number of revertant colonies/3 replicate plates ( $\pm$ S.D.) with one strain of <i>Salmonella typhimurium</i> and one <i>Escherichia coli</i> strain.			
	TA100		WP2uvrA	
	<u>Without S9-mix</u>			
Positive control	1228 $\pm$	56	1672 $\pm$	106
Solvent control	127 $\pm$	5	23 $\pm$	8
1.7	114 $\pm$	17	24 $\pm$	4
5.4	109 $\pm$	28	22 $\pm$	8
17	117 $\pm$	1	22 $\pm$	7
52	111 $\pm$	9	27 $\pm$	3
164	72 $\pm$	4	28 $\pm$	5
512	37 $\pm$	22	25 $\pm$	1
1600	49 $\pm$	10 <sup>NP</sup>	32 $\pm$	7 <sup>NP</sup>
5000	35 $\pm$	3 <sup>n SP</sup>	21 $\pm$	9 <sup>n SP</sup>
	<u>With S9-mix<sup>1</sup></u>			
Positive control	1530 $\pm$	90	461 $\pm$	51
Solvent control	109 $\pm$	4	28 $\pm$	4
1.7	124 $\pm$	5	25 $\pm$	10
5.4	111 $\pm$	2	24 $\pm$	6
17	118 $\pm$	9	22 $\pm$	10
52	129 $\pm$	6	28 $\pm$	1
164	121 $\pm$	11	27 $\pm$	6
512	65 $\pm$	11	27 $\pm$	6
1600	52 $\pm$	9 <sup>NP</sup>	26 $\pm$	3 <sup>NP</sup>
5000	44 $\pm$	6 <sup>n SP</sup>	31 $\pm$	8 <sup>n SP</sup>

<sup>1</sup> Plate incorporation assay (5% S9)

NP No precipitate

SP Slight Precipitate

n Normal bacterial background lawn

## APPENDIX 1 — continued —

**Table 2** Experiment 1: Mutagenic response of MLA-3202 in the *Salmonella typhimurium* reverse mutation assay

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of <i>Salmonella typhimurium</i> .					
	TA1535		TA1537		TA98	
	<u>Without S9-mix</u>					
Positive control	859 ±	39	572 ±	70	1519 ±	87
Solvent control	8 ±	5	5 ±	2	11 ±	1
5.4	5 ±	3	2 ±	3	15 ±	4
17	10 ±	7	4 ±	1	11 ±	6
52	4 ±	4	2 ±	2	12 ±	7
164	2 ±	2	4 ±	1	11 ±	1
512	7 ±	3	4 ±	4	7 ±	5
1600	3 ±	4 <sup>NP</sup>	1 ±	1 <sup>NP</sup>	11 ±	6 <sup>NP</sup>
5000	5 ±	3 <sup>n SP</sup>	3 ±	4 <sup>n SP</sup>	14 ±	4 <sup>n SP</sup>
	<u>With S9-mix<sup>1</sup></u>					
Positive control	297 ±	50	436 ±	36	1429 ±	93
Solvent control	8 ±	3	4 ±	3	19 ±	7
5.4	9 ±	3	8 ±	3	16 ±	2
17	10 ±	2	4 ±	1	16 ±	5
52	10 ±	4	1 ±	1	18 ±	4
164	8 ±	4	5 ±	2	21 ±	8
512	3 ±	4	3 ±	1	14 ±	10
1600	5 ±	2 <sup>NP</sup>	3 ±	2 <sup>NP</sup>	12 ±	8 <sup>NP</sup>
5000	6 ±	4 <sup>n SP</sup>	4 ±	1 <sup>n SP</sup>	9 ±	8 <sup>n SP</sup>

<sup>1</sup> Plate incorporation assay (5% S9)

NP No precipitate

SP Slight Precipitate

n Normal bacterial background lawn

## APPENDIX 1 — continued —

**Table 3** Experiment 2: Mutagenic response of MLA-3202 in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of <i>Salmonella typhimurium</i> and one <i>Escherichia coli</i> strain.									
	TA1535		TA1537		TA98		TA100		WP2uvrA	
	<u>Without S9-mix</u>									
Positive control	946 ±	34	990 ±	80	1873 ±	107	866 ±	45	1585 ±	34
Solvent control	6 ±	4	5 ±	2	14 ±	2	88 ±	28	18 ±	9
492	3 ±	1	4 ±	1	7 ±	2	36 ±	8	12 ±	6
878	4 ±	5	3 ±	2	7 ±	6	23 ±	1	19 ±	1
1568	3 ±	2	2 ±	2	5 ±	3	20 ±	9	16 ±	2
2800	3 ±	2 NP	2 ±	2 NP	5 ±	4 NP	23 ±	4 NP	14 ±	5 NP
5000	3 ±	2 n SP	2 ±	3 n SP	4 ±	1 n SP	19 ±	7 n SP	16 ±	7 n SP
	<u>With S9-mix<sup>1</sup></u>									
Positive control	151 ±	27	590 ±	28	712 ±	20	1447 ±	123	512 ±	15
Solvent control	8 ±	2	11 ±	5	18 ±	7	103 ±	18	25 ±	9
492	4 ±	3	7 ±	2	20 ±	5	68 ±	6	26 ±	3
878	4 ±	1	7 ±	4	17 ±	1	44 ±	4	25 ±	9
1568	6 ±	3	5 ±	1	18 ±	6	40 ±	10	18 ±	4
2800	4 ±	3 NP	8 ±	1 NP	17 ±	3 NP	49 ±	10 NP	15 ±	7 NP
5000	2 ±	1 n SP	7 ±	3 n SP	15 ±	10 n SP	32 ±	11 n SP	15 ±	5 n SP

<sup>1</sup> Plate incorporation assay (10% S9)

NP No precipitate

SP Slight Precipitate

n Normal bacterial background lawn

APPENDIX 1 — continued —

**Table 4 Experiment 3: Mutagenic response of MLA-3202 in the *Salmonella typhimurium* reverse mutation assay**

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of <i>Salmonella typhimurium</i> .			
	TA98		TA100	
	<u>Without S9-mix</u>			
Positive control	1979 ± 104		1207 ± 39	
Solvent control	15 ± 9		99 ± 6	
17	15 ± 4		98 ± 7	
52	20 ± 2		106 ± 6	
164	15 ± 2		82 ± 9	
512	17 ± 3		55 ± 13	
1600	13 ± 7 NP		42 ± 4 NP	
5000	10 ± 5 n SP		37 ± 10 n SP	
	<u>With S9-mix<sup>1</sup></u>			
Positive control			1684 ± 61	
Solvent control			104 ± 6	
17			101 ± 9	
52			92 ± 5	
164			124 ± 24	
512			78 ± 12	
1600			43 ± 8 NP	
5000			36 ± 5 n SP	

<sup>1</sup> Plate incorporation assay (10% S9)  
 NP No precipitate  
 SP Slight Precipitate  
 n Normal bacterial background lawn

**APPENDIX 2 SUPPORTING MATERIALS AND METHOD**

Bacterial background lawn evaluation

The condition of the bacterial background lawn is evaluated (if indicated), both macroscopically and microscopically by using a dissecting microscope (results are normal unless indicated in tables).

Definition	Characteristics
Normal	Distinguished by a healthy microcolony lawn.
Slightly reduced	Distinguished by a slight thinning of the microcolony lawn.
Moderately reduced	Distinguished by a moderate thinning of the microcolony lawn.
Extremely reduced	Distinguished by an extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
Absent	Distinguished by a complete lack of any microcolony background lawn.

Precipitation evaluation

Evidence of test article precipitate on the plates is recorded by addition of the following precipitation definition.

Definition	Characteristics
Slight Precipitate	Distinguished by noticeable precipitate on the plate. However, the precipitate does not influence automated counting of the plate.
Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, requiring the plate to be hand counted.
Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the required hand count difficult.

Evaluation of the reduction in the number of revertants

The reduction in the number of revertant colonies compared to number of revertants in the solvent control is evaluated as follows:

A reduction of 21-40%: slight reduction.

A reduction of 41-60%: moderate reduction.

A reduction of 61-99%: extreme reduction.

If the size of the microcolonies was increased to small colonies due to an extremely reduced background lawn the reduction is evaluated as microcolonies. If no revertant colonies are observed on the plates the reduction is evaluated as a complete lack of revertants.

However, any mean plate count equal to the minimal value of the historical control data range should be considered not toxic.

**APPENDIX 3 DETAILED TABLES**

Individual plate counts; (following pages)

LIST OF ABBREVIATIONS

n	Normal bacterial background lawn
NP	No precipitate
SP	Slight precipitate

**APPENDIX 3 – continued –**

Dose range finding  
Strain TA100

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	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1283	1231	1171	1228 ±	56
solvent control	131	128	122	127 ±	5
1.7	121	95	127	114 ±	17
5.4	78	132	117	109 ±	28
17	116	118	118	117 ±	1
52	101	114	117	111 ±	9
164	76	68	72	72 ±	4
512	58	39	14	37 ±	22
1600	60 NP	41 NP	45 NP	49 ±	10
5000	33 n SP	33 n SP	39 n SP	35 ±	3

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	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1460	1499	1631	1530 ±	90
solvent control	105	113	110	109 ±	4
1.7	125	128	118	124 ±	5
5.4	113	112	109	111 ±	2
17	109	118	127	118 ±	9
52	133	131	122	129 ±	6
164	133	117	112	121 ±	11
512	73	69	53	65 ±	11
1600	58 NP	42 NP	56 NP	52 ±	9
5000	38 n SP	44 n SP	49 n SP	44 ±	6

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**APPENDIX 3 – continued –**

Dose range finding  
Strain WP2uvrA

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	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	1794	1598	1624	1672	$\pm$ 106
solvent control	16	22	31	23	$\pm$ 8
1.7	19	26	26	24	$\pm$ 4
5.4	31	16	18	22	$\pm$ 8
17	29	22	15	22	$\pm$ 7
52	29	23	29	27	$\pm$ 3
164	30	31	22	28	$\pm$ 5
512	24	26	26	25	$\pm$ 1
1600	26 NP	39 NP	30 NP	32	$\pm$ 7
5000	27 n SP	26 n SP	11 n SP	21	$\pm$ 9

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	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	477	404	501	461	$\pm$ 51
solvent control	30	23	30	28	$\pm$ 4
1.7	14	34	27	25	$\pm$ 10
5.4	23	30	19	24	$\pm$ 6
17	31	11	24	22	$\pm$ 10
52	29	27	27	28	$\pm$ 1
164	23	33	24	27	$\pm$ 6
512	26	34	22	27	$\pm$ 6
1600	26 NP	29 NP	23 NP	26	$\pm$ 3
5000	29 n SP	24 n SP	39 n SP	31	$\pm$ 8

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**APPENDIX 3 — continued —**

Experiment 1  
Strain TA1535

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	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	816	892	869	859 $\pm$	39
solvent control	8	12	3	8 $\pm$	5
5.4	4	8	3	5 $\pm$	3
17	7	5	18	10 $\pm$	7
52	1	8	3	4 $\pm$	4
164	3	3	0	2 $\pm$	2
512	5	5	11	7 $\pm$	3
1600	0 NP	7 NP	3 NP	3 $\pm$	4
5000	8 n SP	3 n SP	4 n SP	5 $\pm$	3

---

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	252	351	287	297 $\pm$	50
solvent control	6	11	8	8 $\pm$	3
5.4	10	11	5	9 $\pm$	3
17	10	11	8	10 $\pm$	2
52	7	8	15	10 $\pm$	4
164	8	4	11	8 $\pm$	4
512	0	7	3	3 $\pm$	4
1600	7 NP	4 NP	5 NP	5 $\pm$	2
5000	3 n SP	5 n SP	11 n SP	6 $\pm$	4

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**APPENDIX 3 – continued –**

Experiment 1  
Strain TA1537

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	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	652	528	535	572 ±	70
solvent control	7	4	5	5 ±	2
5.4	0	1	5	2 ±	3
17	5	4	4	4 ±	1
52	1	1	4	2 ±	2
164	3	5	3	4 ±	1
512	8	1	3	4 ±	4
1600	2 NP	0 NP	2 NP	1 ±	1
5000	0 n SP	7 n SP	2 n SP	3 ±	4

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	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	448	464	395	436 ±	36
solvent control	3	8	2	4 ±	3
5.4	10	5	10	8 ±	3
17	4	4	3	4 ±	1
52	0	1	2	1 ±	1
164	3	7	4	5 ±	2
512	3	3	4	3 ±	1
1600	4 NP	1 NP	4 NP	3 ±	2
5000	4 n SP	4 n SP	3 n SP	4 ±	1

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**APPENDIX 3 — continued —**

Experiment 1  
Strain TA98

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	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	1522	1431	1605	1519	$\pm$ 87
solvent control	11	11	12	11	$\pm$ 1
5.4	11	15	18	15	$\pm$ 4
17	4	15	15	11	$\pm$ 6
52	18	5	12	12	$\pm$ 7
164	10	10	12	11	$\pm$ 1
512	12	3	7	7	$\pm$ 5
1600	14 NP	15 NP	5 NP	11	$\pm$ 6
5000	14 n SP	11 n SP	18 n SP	14	$\pm$ 4

---

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	1383	1536	1367	1429	$\pm$ 93
solvent control	17	27	14	19	$\pm$ 7
5.4	14	15	18	16	$\pm$ 2
17	11	20	18	16	$\pm$ 5
52	15	23	16	18	$\pm$ 4
164	30	18	15	21	$\pm$ 8
512	4	14	24	14	$\pm$ 10
1600	14 NP	19 NP	3 NP	12	$\pm$ 8
5000	0 n SP	15 n SP	12 n SP	9	$\pm$ 8

---

**APPENDIX 3 — continued —**

Experiment 2  
Strain TA1535

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	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	979	911	948	946 $\pm$	34
solvent control	4	11	3	6 $\pm$	4
492	3	3	4	3 $\pm$	1
878	0	9	4	4 $\pm$	5
1568	4	1	3	3 $\pm$	2
2800	4 NP	4 NP	1 NP	3 $\pm$	2
5000	4 n SP	1 n SP	3 n SP	3 $\pm$	2

---

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	125	150	178	151 $\pm$	27
solvent control	7	7	10	8 $\pm$	2
492	7	1	3	4 $\pm$	3
878	3	5	3	4 $\pm$	1
1568	4	4	10	6 $\pm$	3
2800	1 NP	7 NP	4 NP	4 $\pm$	3
5000	3 n SP	1 n SP	1 n SP	2 $\pm$	1

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**APPENDIX 3 — continued —**

Experiment 2  
Strain TA1537

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	WITHOUT S9-MIX			MEAN	SD
plate	1	2	3		
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	1011	1057	902	990 $\pm$	80
solvent control	4	5	7	5 $\pm$	2
492	5	5	3	4 $\pm$	1
878	1	4	3	3 $\pm$	2
1568	4	3	0	2 $\pm$	2
2800	1 NP	1 NP	5 NP	2 $\pm$	2
5000	0 n SP	5 n SP	1 n SP	2 $\pm$	3

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	WITH S9-MIX			MEAN	SD
plate	1	2	3		
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	558	611	600	590 $\pm$	28
solvent control	5	15	12	11 $\pm$	5
492	8	7	5	7 $\pm$	2
878	3	10	7	7 $\pm$	4
1568	5	4	5	5 $\pm$	1
2800	8 NP	7 NP	8 NP	8 $\pm$	1
5000	7 n SP	10 n SP	5 n SP	7 $\pm$	3

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**APPENDIX 3 — continued —**

Experiment 2  
Strain TA98

---

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	1966	1896	1756	1873	$\pm$ 107
solvent control	15	12	15	14	$\pm$ 2
492	8	8	5	7	$\pm$ 2
878	10	0	10	7	$\pm$ 6
1568	8	5	3	5	$\pm$ 3
2800	1 NP	5 NP	8 NP	5	$\pm$ 4
5000	4 n SP	4 n SP	3 n SP	4	$\pm$ 1

---

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	698	702	735	712	$\pm$ 20
solvent control	26	12	15	18	$\pm$ 7
492	16	19	25	20	$\pm$ 5
878	18	16	16	17	$\pm$ 1
1568	14	15	24	18	$\pm$ 6
2800	20 NP	14 NP	16 NP	17	$\pm$ 3
5000	5 n SP	16 n SP	24 n SP	15	$\pm$ 10

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**APPENDIX 3 — continued —**

Experiment 2  
Strain TA100

---

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	835	917	845	866 $\pm$	45
solvent control	120	71	72	88 $\pm$	28
492	44	29	34	36 $\pm$	8
878	22	22	24	23 $\pm$	1
1568	19	30	12	20 $\pm$	9
2800	22 NP	27 NP	19 NP	23 $\pm$	4
5000	14 n SP	15 n SP	27 n SP	19 $\pm$	7

---

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	1373	1378	1589	1447 $\pm$	123
solvent control	83	117	110	103 $\pm$	18
492	71	61	71	68 $\pm$	6
878	44	41	48	44 $\pm$	4
1568	39	30	50	40 $\pm$	10
2800	61 NP	42 NP	45 NP	49 $\pm$	10
5000	20 n SP	34 n SP	42 n SP	32 $\pm$	11

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**APPENDIX 3 — continued —**

Experiment 2  
Strain WP2uvrA

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	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	1611	1597	1547	1585	$\pm$ 34
solvent control	8	20	25	18	$\pm$ 9
492	15	5	16	12	$\pm$ 6
878	20	19	18	19	$\pm$ 1
1568	14	18	15	16	$\pm$ 2
2800	16 NP	18 NP	8 NP	14	$\pm$ 5
5000	19 n SP	8 n SP	22 n SP	16	$\pm$ 7

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	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	501	529	507	512	$\pm$ 15
solvent control	20	35	20	25	$\pm$ 9
492	23	29	26	26	$\pm$ 3
878	15	31	29	25	$\pm$ 9
1568	15	22	16	18	$\pm$ 4
2800	23 NP	12 NP	11 NP	15	$\pm$ 7
5000	10 n SP	20 n SP	15 n SP	15	$\pm$ 5

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**APPENDIX 3 — continued —**

Experiment 3  
Strain TA98

---

	plate	WITHOUT S9-MIX			MEAN	SD
	1	2	3			
dose ( $\mu\text{g}/\text{plate}$ )						
positive control	1907	2098	1933	1979	$\pm$ 104	
solvent control	24	7	14	15	$\pm$ 9	
17	19	14	12	15	$\pm$ 4	
52	19	18	22	20	$\pm$ 2	
164	14	18	14	15	$\pm$ 2	
512	20	15	15	17	$\pm$ 3	
1600	11 NP	20 NP	7 NP	13	$\pm$ 7	
5000	8 n SP	16 n SP	7 n SP	10	$\pm$ 5	

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**APPENDIX 3 – continued –**

Experiment 3  
Strain TA100

---

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	1208	1245	1167	1207	$\pm$ 39
solvent control	95	106	97	99	$\pm$ 6
17	101	90	102	98	$\pm$ 7
52	103	103	113	106	$\pm$ 6
164	84	72	90	82	$\pm$ 9
512	57	67	42	55	$\pm$ 13
1600	44 NP	44 NP	37 NP	42	$\pm$ 4
5000	46 n SP	39 n SP	27 n SP	37	$\pm$ 10

---

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose ( $\mu\text{g}/\text{plate}$ )					
positive control	1703	1734	1616	1684	$\pm$ 61
solvent control	109	98	106	104	$\pm$ 6
17	105	107	91	101	$\pm$ 9
52	97	91	87	92	$\pm$ 5
164	151	107	113	124	$\pm$ 24
512	82	65	87	78	$\pm$ 12
1600	50 NP	44 NP	35 NP	43	$\pm$ 8
5000	42 n SP	34 n SP	33 n SP	36	$\pm$ 5

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**APPENDIX 4 HISTORICAL CONTROL DATA OF THE SOLVENT CONTROL**

	TA1535		TA1537		TA98		TA100		WP2uvrA	
S9-mix	-	+	-	+	-	+	-	+	-	+
Range	5 - 36	3 - 34	3 - 25	3 - 28	9 - 50	9 - 57	63 - 153	60 - 156	13 - 68	12 - 70
Mean	17	14	7	9	18	26	104	105	28	34
SD	6	5	3	3	6	7	17	17	7	7
n	1644	1716	1425	1443	1707	1730	1725	1739	1368	1404

SD = Standard deviation

n = Number of observations

Historical control data from experiments performed between November 2013 and January 2016.

**APPENDIX 5 HISTORICAL CONTROL DATA OF THE POSITIVE CONTROL ITEMS**

	TA1535		TA1537		TA98	
S9-mix	-	+	-	+	-	+
Range	78 - 1932	81 - 1332	62 – 1565	55 – 1112	347 – 1967	261 - 1885
Mean	791	234	662	409	976	821
SD	261	98	206	126	251	298
n	1732	1737	1409	1428	1721	1737

	TA100		WP2uvrA	
S9-mix	-	+	-	+
Range	549 – 1798	640 - 2760	123 – 1958	85 - 1390
Mean	914	1387	1367	261
SD	150	324	276	276
n	1734	1752	1373	1404

SD = Standard deviation

n = Number of observations

Historical control data from experiments performed between November 2013 and January 2016.

## APPENDIX 6 CERTIFICATE OF ANALYSIS



Chemtura Corporation  
12 Spencer St  
Naugatuck, CT 06770

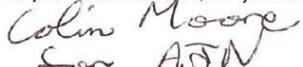
Analytical Services  
[www.chemtura.com](http://www.chemtura.com)

## Certificate of Purity

Customer: Support for Toxicology Studies  
Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)  
Physical Appearance: Liquid  
CAS No.: 1454803-04-3  
Ref. or Lot Number: RC-1045  
Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	C <sub>24</sub> H <sub>47</sub> NO <sub>3</sub>	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	C <sub>22</sub> H <sub>45</sub> NO <sub>3</sub>	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	C <sub>24</sub> H <sub>45</sub> NO <sub>3</sub>	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	C <sub>24</sub> H <sub>49</sub> NO <sub>3</sub>	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	C <sub>22</sub> H <sub>43</sub> NO <sub>3</sub>	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	C <sub>26</sub> H <sub>45</sub> NO <sub>3</sub>	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	C <sub>24</sub> H <sub>43</sub> NO <sub>3</sub>	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	C18:1 (oleic) acid
1.1	421.4	C <sub>26</sub> H <sub>47</sub> NO <sub>3</sub>	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

  
 Blake Lewis  
 Analytical REACH Scientist, Analytical Services  
 Date 3/7/16

  
 Albert J. Nitowski  
 Sr. Technology Manager  
 Analytical and Lab Support Services  
 Date 3/7/16